Chapter 3

MATERIALS AND METHODS
MATERIALS AND METHODS

3.1.1. Chemicals and reagents:

$^{3}$H thymidine (Amersham Int. U.K.), Cell titre 96™ non-radioactive cell proliferation assay kit (Promega, Madison), HEPES (Sigma, M.O), murine monoclonal antibody (4D5) against c-neu protein (Genentech Inc. CA), trypsin - EDTA solution (Biological Industries, Israel), glass fibre filter strips (Cambridge Tech. Inc. MA), fetal calf serum (GIBCO, New York), Dulbecco's modified Eagle's medium (DMEM) (GIBCO), RPMI 1640 medium (GIBCO), bovine serum albumin (Sigma) and Tris-HCL (Sigma) were procured for the study. Other chemicals used for the study were of analytical grade.

3.1.2. Plastic wares:

Disposable plastic wares like flasks, petri-dishes, tissue culture plates, pipettes, culture tubes etc. were procured from Corning glass works, New York and Nunc, Denmark.

3.1.3. Cell lines:

The LNCaP cell line, sensitive to androgen, was established from a metastatic lymph node (Horoszewicz et al. 1983). Androgen independent DU 145 human prostate cancer cell line was established from a metastatic brain lesion of a Caucasian male (Mickey et al. 1977). These cell lines were obtained from the American Type Culture Collection (ATCC). LNCaP cells were grown in RPMI 1640 medium (GIBCO) supplemented with 7% heat inactivated fetal calf serum (GIBCO), penicillin (100 units/ml.) and streptomycin (100 µg/ml) at 37°C in a humidified atmosphere of 5% CO₂ in air. DU 145 cells were grown in Dulbecco's modified Eagle's medium
(GIBCO) supplemented with 10% heat inactivated fetal calf serum, penicillin and streptomycin. HEp-2 cells (laryngeal carcinoma cell line) were received from National Tissue Culture Facility, Pune and were grown in DMEM supplemented with 10% heat inactivated fetal calf serum, penicillin and streptomycin as mentioned in case of other cell lines.

3.1.4. Antibodies:

The following antibodies were used for immunohistochemical studies:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Anti-TGF-α</td>
<td>Monoclonal</td>
<td>Oncogene Science Inc.</td>
</tr>
<tr>
<td>3. Anti-TGF-β</td>
<td>Polyclonal</td>
<td>British Biotech. Ltd.</td>
</tr>
<tr>
<td>5. Anti-FGF-basic</td>
<td>Polyclonal</td>
<td>Oncogene Science Inc.</td>
</tr>
<tr>
<td>8. Anti-PSA</td>
<td>Monoclonal</td>
<td>Dakopatts</td>
</tr>
<tr>
<td>9. Anti-PSAP</td>
<td>Polyclonal</td>
<td>Dakopatts</td>
</tr>
</tbody>
</table>

* A generous gift from Dr. U. Rodeck

3.1.5. Human prostatic tissues:

Prostate tissues were taken from patients who underwent transurethral prostatic resection (TURP) and suprapubic prostatectomy at the All India Institute of Medical Sciences (AIIMS), New Delhi. They were within the age
group of 40 to 91 years and were suffering from either benign prostatic hyperplasia or adenocarcinoma of the prostate for a period ranging from few months to eight years. Clinical grading of the prostate size, which estimates the degree of protrusion of the gland into rectum as estimated by digital per rectal examination, was done as per the established method (Barnes et al. 1959). Soon after surgical removal, the prostate tissues were washed in ice-cold phosphate buffered saline (PBS 0.1M, pH 7.4). The charred pieces having been eliminated or trimmed off, these were fixed in 10% buffered formalin and picric acid containing Zamboni fixative (0.1M). After 22-24 hrs. of fixation the tissues were processed in a histomatic tissue processor (Fisher Scientific, Pittsburgh, USA) and 5-6μm sections were taken in an autocut rotary microtome (Reichert - Jung, Germany). Few large undamaged prostatic chips were frozen in isopentane (BDH, England) previously cooled with liquid nitrogen, wrapped in foil and stored in liquid nitrogen. 5-6μm frozen sections were cut after embedding in the OCT matrix (Histolab - cytolab, UK) in a cryocut-2800 Frigocut-N (Reichert-Jung). After air drying at room temperature frozen sections were stored at -20°C and used within a week for immunohistochemical studies. These were fixed in chilled acetone for 10 mins. prior to staining.

3.2. Immunohistochemistry:

Tissue sections were stained by avidin - biotin complex (ABC) peroxidase, peroxidase anti-peroxidase (PAP) (Hsu et al. 1981) and indirect peroxidase methods. For antibodies against TGF-α, EGFR, TGF-β, aFGF, bFGF and PSA avidin - biotin complex peroxidase method was employed using Vectastain, elite ABC kit (Vector Lab. Inc., Burlingame). For PSAP antibodies peroxidase anti-peroxidase method was employed using universal
DAKO PAP Kit (Dakopatts, Denmark). Goat polyclonal antibodies against EGF were studied by the indirect peroxidase method.

3.2.1. Avidin-biotin complex (ABC) peroxidase method:

Sections were deparaffinized in xylene, cleared and hydrated in descending grades of propanol. After rinsing in distilled water for 5 mins, sections were incubated with H₂O₂-methanol mixture followed by washing with 0.1M PBS, pH 7.4 for 20 mins. Sections were then incubated for 20 mins. in a humidified chamber with 0.1M PBS, pH 7.4 containing 1.5% normal horse serum. Excess serum was blotted and primary antibody at the appropriate dilution in 0.1M PBS, pH 7.4 was layered over the sections followed by incubation overnight for 10-12 hrs. in a humidified chamber at room temperature. Sections were washed with 0.1M PBS, pH 7.4 for 30 mins. (three changes). Biotinylated anti-mouse IgG (1.5%) in 0.1M PBS, pH 7.4 containing 1.5% normal horse serum was layered over the sections and incubated in a humidified chamber for 30 mins. Sections were washed with 0.1 M PBS, pH 7.4 for 50 mins. (five changes) followed by incubation of the sections with avidin-biotin complex peroxidase for 30 mins. in a humidified chamber. Slides were then washed for 30 mins. with 0.1M PBS, pH 7.4 (three changes). Endogenous peroxidase quenching was carried out at this stage in case of frozen sections. The reaction was visualized with a diaminobenzidine substrate solution [100 ml Strait buffer (8.5 g ammonium acetate, 1.1 g calcium chloride, distilled water upto 1000 ml) pH 5.1; 10 mg 3-3' diaminobenzidine (Sigma, M.O) and 8μl of H₂O₂ (30% w/v) - mixed and filtered through Whatman filter paper]. Control parallel sections were stained identically except that the primary antibody was either omitted or replaced by an unrelated monoclonal antibody either specific for *Shigella flex-
neri Y (kindly provided by Dr. Z. A. Ahmed, ICDDR, Bangladesh) or Salmonella typhi. Slides were counterstained using Harris hematoxylin.

3.2.2. Peroxidase anti-peroxidase (PAP) method:

Paraffin sections were incubated at 56 - 60°C for 30 mins. and transferred immediately to xylene bath (3 mins), followed by another 3 mins. in fresh xylene. Sections were cleared and hydrated in descending grades of propanol to distilled water and incubated with 3% (w/v) hydrogen peroxide in water for 15 mins. at room temperature. Slides were rinsed in distilled water and placed in 0.05M Tris buffer, pH 7.6 for 5 mins. Sections were then incubated with normal swine serum (30 mins., room temp.). Excess serum was tapped off and rabbit polyclonal antibody against PSAP (1 : 100) applied over the sections and incubated overnight 10 - 12 hrs, at room temp. in a humidified chamber. After slides were rinsed followed by washing in 0.05M Tris buffer, pH 7.6 with 3 changes (10 mins. each), swine anti-rabbit immunoglobulins were applied over the sections and incubated (1 hr., room temp.) in a humidified chamber. Slides were washed in 0.05M Tris buffer, pH 7.6 (5 changes, 10 mins. each), and sections were covered with rabbit PAP and incubated (1 hr., room temp.) in a humidified chamber. After washing in 0.05M Tris buffer, pH 7.6 (3 changes, 10 mins. each), sections were exposed to substrate solution [2ml substrate buffer, 50 μl amino-ethylcarbazole and 50 μl H₂O₂ (30% w/v)] for 20 - 30 mins. for colour development. Sections were counterstained with Mayer's hematoxylin and mounted with glycerol - gelly. Control parallel sections were stained similarly except that the primary antibody step was omitted.
3.2.3. Indirect peroxidase method:

After sections were deparaffinized, hydrated and endogenous peroxidase quenched as mentioned under ABC technique, these were incubated with normal rabbit serum (1:5 in 0.1M PBS, pH 7.4) for 30 mins. at room temperature. Excess serum was blotted and primary antibody (goat anti-EGF) was layered over the sections and incubated for 10 -12 hours at room temperature in a humidified chamber. Sections were thoroughly washed with 0.1M PBS, pH 7.4 (3 changes ·10 mins. each) and rabbit anti-goat IgG conjugated to HRP (1:50 in 0.1M PBS, pH 7.4) was layered over the sections prior to incubation (1 hr., room temp.) in a humidified chamber. The reaction was visualized using a substrate solution and counterstained as described for the ABC technique. Control parallel sections were stained identically except that the primary antibody was replaced by PBS.

3.2.4. Scoring method:

For analysis and semi-quantitation of the immunohistochemical staining, the following criteria were considered; the type of cells (secretory, basal, stromal and cancerous) showing positivity; the percentage of cells showing positive reaction (0 - 10% = 0, 11 - 25% = 1, 26 - 50% = 2, 51 - 75% = 3 and 76 - 100% = 4) and the intensity of staining (ws = weak; ms = moderate and ss = strong staining).

3.3.1. PSA assay:

Serum PSA concentration was estimated using an immunoenzymatic assay kit (Tandem - E, Hybritech Inc. CA). Samples were incubated separately with plastic beads (solid phase) coated with a monoclonal antibody directed towards a unique site on the PSA molecule and with an enzyme -
labelled monoclonal antibody directed against a distinct antigenic site. Following the formation of the solid phase/PSA/labelled antibody sandwich, the bead was washed to remove unbound labelled antibody and was then incubated with an enzyme substrate. The amount of substrate turnover was determined colorimetrically by measuring the absorbance of the quenching reaction at 405 nm. The calculation of PSA concentration in the specimen was based on concurrent testing of the PSA standards supplied in the assay kit. Controls consisted of samples from apparently healthy young volunteers within the age group of 18 - 25 years and from patients suffering from tumours of non-prostatic origin (urinary bladder cancer) who had no history of prostate enlargement. Normal PSA values range between 0 to 3.9 µg per liter. A value > 4.0 µg per liter was considered abnormal.

3.3.2. PSAP assay:

Concentrations of PSAP in serum were also tested using an immunoassay kit (Tandem - E, Hybritech Inc. CA). It is a solid phase, two-site immunoenzymatic assay. Serum samples containing PSAP were reacted with plastic beads (solid phase) coated with a monoclonal antibody against PSAP and with an enzyme labelled second monoclonal antibody. These two monoclonals recognize two distinctly different antigenic sites on PSAP molecule. Following formation of solid phase/PSAP/labelled antibody sandwich the bead was washed to remove unbound labelled antibody and incubated with an enzyme substrate. The amount of substrate turnover was determined colorimetrically by measuring absorbance of the quenched reaction. Absorbance was directly proportional to the concentration of PSAP in the sample. The calculation was based on simultaneous testing of PSAP
standards. The normal serum concentration of prostatic acid phosphatase in men ranges from 0 - 5 ng/ml.

3.3.3. Serum testosterone assay:

7-10 ml of blood samples from each patient was collected prior to initiation of surgical intervention. Serum was separated and stored at -70°C until analysis. Serum testosterone was estimated by radioimmunoassay using WHO Matched Assay reagents and following the protocol described in the WHO Manual (1992). Briefly, 100 µl serum (diluted 1:5 in 0.1M phosphate buffer containing 0.88% NaCl, 0.01% thiomersal, 0.1% gelatin) was mixed with 2 ml chilled ether. After vortex mixing for 2 mins. the aqueous phase was frozen in liquid nitrogen. The supernatant was decanted and ether was allowed to evaporate. 0.5 ml of assay buffer was added to each tube and incubated at 40°C in a waterbath for 30 mins. Standards ranged from 320 pg/tube to 10 pg/tube of testosterone. The tubes were then vortex mixed and 100µl each of appropriately diluted anti-testosterone and ³H-testosterone working solution (10,000 cpm) was added. After incubation for 18 - 20 hrs. at 4°C, 0.2 ml of cold dextran - charcoal reagent [0.0625g dextran and 0.625g charcoal per 100 ml assay buffer] was added to each tube and incubated for 30 mins. Tubes were centrifused at 2000 rpm. for 10 mins, supernatants were decanted into vials and 4 ml scintillation cocktail [4g 2, 5-diphenyloxazole (Sigma, MO), 0.12 g dimethyl POPOP (Sigma, MO), toluene (sulphur free) 1 lit] was added to each tube, equilibriated for 2 hrs. and counted in a LKB beta counter.

3.4. Purification of Prostate Specific Antigen:

PSA was purified from seminal plasma by a modified method of Wang et al (1979). Human semen samples were obtained from the volunteers
attending the infertility clinic at the All India Institute of Medical Sciences, New Delhi. They had no apparent indication of genito-urinary infections. Semen samples were pooled, allowed to liquify and sperm separated by centrifugation (2000 rpm, 10 mins.) at room temperature. The seminal plasma was clarified by centrifugation (36,000 xg, 20 mins., 4°C) and stored frozen at -70°C until use.

3.4.1. Ion exchange chromatography:

In a typical batch, 10 to 15 ml of seminal plasma double diluted with an equal volume of 10 mM Tris-HCl, pH 7.4 and serially precipitated with 33.3%, 50%, 66.6% and 75% of saturated ammonium sulphate. The 66.6% precipitate was found to contain the major portion of PSA. This precipitate was washed with the same percentage of ammonium sulphate, dissolved in minimum volume of 10mM Tris-HCl, pH 8.0 and dialysed against the same buffer. The dialysed protein was subjected to centrifugation at 19,000 x g, for 10 mins., at 4°C and loaded onto an ion exchange column (2.6 x 30 cm), DEAE-Sepharose fast-flow (Pharmacia, Uppsala, Sweden) previously equilibrated with 10mM Tris-HCl, pH 8.0. Proteins were eluted with a gradient of the same buffer containing 0.3M NaCl. Figure 1 shows the elution profile from the above column. The fractions positive for PSA in an immunoenzymatic assay were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis made according to Laemmli (1970), to confirm its presence (Fig.2). Fractions (No.17-20) showed high concentration of PSA. As the fractions 19 and 20 contained high amounts of other proteins only fractions 17 and 18 were pooled and concentrated to around 2 ml by Amicon assembly.
Fig. 1: Elution profile of PSA on a DEAE-Sepharose fast-flow column. 66.6% ammonium sulphate precipitate of seminal plasma was used for ion exchange chromatography.
Fig. 2: SDS-PAGE analysis of fractions collected from ion exchange chromatography. 5 μl fractions No. 15 to 21 diluted in equal volume of sample buffer loaded separately in the wells. Presence of PSA is detectable in fractions 16, 17, 18, 19 and 20. Molecular weight markers were loaded in the lane designated by m.
3.4.2. Gel filtration:

The concentrated protein obtained from ion-exchange chromatography was then loaded onto a gel filtration column (1.5 x 80 cm), Sepacryl S-100 (Pharmacia, Uppsala, Sweden) along with 50mM Tris-HCl containing 0.15M NaCl, pH 7.4. Figure 3 shows the elution profile from the above column. The fractions, 2ml each thus collected were tested for PSA using an immunoenzymatic assay and the peak fractions (No. 37 to 46) were tested for purity by SDS-PAGE (Fig.4).

The gels were stained with Coomassie blue (0.25% in 40% methanol, 10% glacial acetic acid and 50% distilled water) for 45-60 minutes, destained overnight using the destaining solution (40% methanol and 10% glacial acetic acid in distilled water) on a rocker and dried in a gel dryer.

3.4.3. Western blot of fractions:

Fractions collected after gel filtration were subjected to SDS-PAGE. Each fraction was mixed with equal volume of SDS gel sample buffer, incubated in a boiling waterbath for 5 mins. and 10μl of each sample was loaded per well. The protein was transferred to immobilon-P PVDF membrane (0.45μ) (Millipore Corporation) at constant voltage of 10 volts overnight at 4°C (Towbin et al. 1979) using a Bio-Rad electroblotting apparatus. The membrane was blocked for 60 minutes with Tris-buffered saline containing Tween 20 (10 mM Tris-HCl, pH 8.0; 150 mM NaCl and 0.05% Tween 20, TBST) and 1% milk solution at room temperature. The membrane was washed twice by soaking in TBST for 10 minutes each and incubated for 60 minutes with anti-PSA mouse monoclonal antibody (10 μg/ml) diluted in TBST containing 0.5% milk solution. The membrane was
Fig. 3: Elution profile of Sephacryl S-100 column when pooled fractions of 17 and 18 from DEAE-SEPHAROSE column was loaded. Arrow indicates the initiation of elution of PSA represented by the hump.
washed with TBST (3 changes, 10 mins. each) followed by incubation for 60 minutes at room temperature with anti-mouse IgG-HRP diluted (1:50) in TBST. The membrane was again washed with TBST (5 changes, 10 minutes each). Colour was developed using a substrate solution [30 mg 4-chloro-1-naphthol (Sigma, MO); 10 ml methanol; 40 ml 0.1M PBS, pH 7.4 and 50 µl H₂O₂ (30% w/v)]. Primary antibody was replaced by TBST in control runs. Anti-PSA monoclonal antibody revealed the presence of PSA in fractions 39 to 46 (Fig.5). These fractions were pooled. This pooled fraction along with fractions obtained at various stages of purification were subjected to SDS-PAGE followed by silver staining to compare and assess the purity of the protein. As shown in figure 6 this final product on silver staining gave a single band at 34 kDa region corresponding to a protein at the same region in the seminal plasma and the pooled fractions from ion-exchange chromatography.

Silver staining of gels was carried out using a Phast gel silver kit (Pharmacia, USA). Gels were submerged in destaining solution for 5 minutes, the solution was poured out and fresh solution was added for another 5 mins. These were then exposed to a 5% glutaraldehyde solution for 10 minutes after which two incubations of 5 mins. each with destaining solutions were carried out. Gels were then washed thrice with distilled water 5 minutes per wash and exposed to a 0.4% silver nitrate solution for 8 minutes followed by washing in distilled water four times, 30 sec. per wash. They are then treated with developer (2.5% sodium carbonate) for 5 to 10 minutes till the colour appeared. Gels were washed quickly for 3-4 times in distilled water, before they were left in 5% glycerol solution overnight and dried. Estimation of protein was carried out by the method of Lowry et al (1951) using bovine serum albumin as standard.
**Fig. 4**: Fractions as represented by the numbers from 37 to 46 from gel filtration were analysed by SDS-PAGE. Lane in the extreme left was loaded with the molecular weight markers. Fractions 39 to 46 contained PSA as indicated by arrow. (Top left)

**Fig. 5**: Western blot of the fractions (37 to 46) from gel filtration. 34kD protein PSA (arrow) was detected in the fractions from 39 to 46 by a monoclonal antibody. (Top right)

**Fig. 6**: Characterization of PSA purification products by SDS-PAGE followed by silver staining. Lanes M, molecular weight markers; lane SP, whole seminal plasma; lane ASP, 66.6% ammonium sulphate precipitate; lane DS, DEAE-SEPHAROSE fraction; lane GF, purified PSA after gel filtration. (bottom)
3.5.1. Raising of anti-PSA antibodies:

100 μg PSA in 1ml 50mM PBS, pH 7.4, was emulsified with 1 ml complete Freund's adjuvant and injected intradermally into each goat. Two subsequent injections 50 μg PSA each in incomplete Freund's adjuvant (IFA) were given at monthly intervals followed two months later by last injection (25 ug of PSA in IFA). Animals were bled at intervals and serum samples were assayed for antibodies using an ELISA.

PSA in phosphate buffered saline (50 mM PBS, pH 7.4) was coated (0.5 μg/well) to 96-well plate (Maxisorb. Nunc., Denmark) and incubated at 37°C for one hour followed by overnight incubation at 4°C. The plates were washed once with PBS-Tween (50mM PBS, pH 7.4 containing 0.2% Tween 20). Serum samples at various dilutions in the same buffer, were dispensed into the wells and incubated for 1hr. at 37°C in a humidified chamber. Plates were washed three times with PBS-Tween followed by incubation for 1 hr. at 37°C with anti-goat immunoglobulin conjugated to HRP, diluted appropriately in washing buffer. After washing five times with PBS-Tween, colour was developed with 100 μl of chromogen solution (O-phenylene diamine 0.5mg/ml, 0.03% H₂O₂ in 0.15M citrate - phosphate buffer, pH 5.5) for 10 minutes in the dark. The reaction was stopped with 50 μl of 5N H₂SO₄ and absorbance read at 490 nm using an ELISA reader (Molecular Devices, USA). Anti-PSA Ig were quantified by ELISA using goat Ig as standard.

3.5.2. Purification of antibodies:

Purification of anti-PSA and anti-LHRH antibodies were carried out using a Protein G-Sepharose column (5 ml). The column was washed with 50 mM Tris-HCl, pH 7.4, containing 0.25 M NaCl. The diluted (1:4) serum
sample or ascitic fluid (anti-LHRH antibody) was loaded onto the column and immunoglobulins were eluted with 1N acetic acid containing 0.5M NaCl. Fractions were neutralized with 2M Tris-HCl, pH 7.4, concentrated on filter assembly using Amicon YM 10 membrane, dialysed and protein content estimated.

3.5.3. Western blot of tissue homogenates for PSA:

Prostatic tissue samples collected from BPH patients and kept frozen in liquid nitrogen were homogenized in 2 volumes (w/v) of a modified SBN lysis buffer [50 mM Tris-HCl, pH 7.5; 0.15 M NaCl; 10% glycerol; 1% Nonidet P40 (Sigma, M.O); 1mM EGTA (Sigma); 0.01% leupeptin (Sigma); 0.01% aprotinin (Sigma); 2mM PMSF (Sigma)] as described by Peles et al (1991). Cell lysates were centrifuged at 12,000xg for 10 mins. at 4°C. The supernatnat was aliquoted and either kept at -70°C until analysed or subjected to electrophoresis as described previously. Proteins thus separated were transferred onto immobilon-P PVDF membranes blotted with anti-PSA polyclonal antibodies (20 µg/ml) and a monoclonal antibody (10 µg/ml). Both polyclonal antibodies (Fig.7) and monoclonal antibody (Fig.8) recognized 34 kDa band in the tissue homogenates from hyperplastic human prostates. A few benign prostate glands were studied for immunocytochemistry and homogenates immunoblotted using anti-PSA antibodies. The immunohistochemical reaction was comparable to the Western blot analysis in all the samples tested (Fig.9).

3.5.4. Screening of human tissues for PSA:

Tissues of young males were studied for expression of PSA immunohistochemically using purified goat anti-PSA IgG. Tissues viz., liver,
Fig. 7: Western blot of BPH tissue homogenates with anti-PSA polyclonal antibody. Lane psa, purified PSA; lane immediately next to psa lane, molecular weight markers; lanes 46 to 3, prostate tissue homogenates from patients. Antibody recognized PSA as indicated by arrow in all the samples except 34.
Fig. 8: Western blot of hyperplastic human prostate tissue homogenates from patients with an anti-PSA monoclonal antibody. Lane psa, purified PSA; lanes 31 to 46, prostate tissue homogenates from patients. Antibody recognizes PSA in all the samples as indicated by arrow.
Fig. 9: Comparative analysis of immunoblot (IB) and immunohistochemistry (IHC) employing anti-PSA IgG in six hyperplastic prostates from human patients. Intensity of immunohistochemical reaction is fairly comparable to the thickness of the band recognized by the antibody in immunoblot analysis of same tissue homogenate using a constant volume.
lung, kidney, adrenal, thyroid, heart, lymphnode, skeletal muscle, pancreas, salivary gland, prostate, seminal vesicle, testis, epididymis, skin, spleen, stomach and brain were collected during autopsies of accident cases conducted at the Forensic Science Department, All India Institute of Medical Sciences, New Delhi. The tissues were preserved in 10% buffered formalin. Paraffin embedded tissues were sectioned at 5 micron intervals. Slides from a few BPH and CaP cases were also included for immunohistochemical staining of PSA. Following indirect immunoperoxidase method sections were stained using anti-PSA IgG at the concentration of 20 μg/ml of PBS (0.1M, pH 7.4). The staining protocol has been described earlier. PSA could not be detected in any of the tissues of non-prostatic origin. PSA detected in all three normal human prostate glands was low in comparison to hyperplastic and malignant prostates.

3.6. Immunocytochemistry of cells for PSA, c-neu protein and LHRH:

LNCaP and DU 145 cells were grown on coverslips in 24-well tissue culture plates for 72 hours. The coverslips were washed three times with 0.1M PBS, pH 7.4 and fixed at room temperature with 1% paraformaldehyde for 30 min. The fixed cells were washed, presaturated in PBS containing 1% BSA and 5% normal goat serum. The cells were incubated with either anti-PSA (1:40) or anti-c-neu (1:50) or anti-LHRH (1:100) monoclonal antibodies for one hour at room temperature followed by washing five times with 0.1M PBS, pH 7.4. Gold conjugated goat anti-mouse IgG (1:50) (British Biotech. Ltd., Oxford) was used as a secondary antibody and incubated for 45 minutes at room temperature. The coverslips were washed five times with 0.1M PBS, pH 7.4 followed by washing two times with distilled water and silver enhanced using a kit (British Biotech. Ltd., Oxford). Cells where primary antibody step was omitted served as controls.
3.7. Studies with anti-c-neu antibody (4D5) on growth of prostatic carcinoma cells.

5 X 10^3 cells (in 100 µl) from the cell suspensions (5 X 10^4 cells/ml) of LNCaP and DU 145 cells grown in tissue culture flasks, were dispensed in 96-wells tissue culture plates. The cell suspensions were made after harvesting the cells with 0.02% EDTA in normal saline at the log phase of growth. The culture plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 24 hrs. Anti-c-neu antibody (2.5 µg/ml) diluted in media and 40 µl of medium each containing 0.156, 0.312, 0.625, 1.25 and 2.5 µg of 4D5 monoclonal antibody was dispensed in each well and the culture plates were incubated at 37°C in a CO_2 incubator for 48 hrs. 15 µl of dye solution (non-radioactive cell proliferation assay, Promega) was dispensed into each well and incubated at 37°C for 4 hrs. 100 µl of solubilization solution was dispensed into each well and incubated at room temperature overnight (10 - 12 hrs.). Absorbance was recorded at 570 nm in an ELISA reader with low speed shaking (30 secs.). The experiments were repeated in an identical manner except the addition of higher doses of 4D5 monoclonal antibody viz. 0.1, 1, 10 and 100 µg in 100 µl medium.


3.8.1.1. a) ^3[H] thymidine incorporation assay:

The procedure described by Ihle et al. (1982) has been followed for this assay. LNCaP and DU 145 cells were grown in tissue culture flasks separately. The cells were harvested at the log phase of growth with trypsin-EDTA solution and suspended in media supplemented with FCS and antibiotic.
mixture to the final cell density of $5 \times 10^4$ cells per ml. This cell suspension was dispensed into 96-well tissue culture plates 100 $\mu$l per well, incubated at 37°C in a humidified atmosphere of 5% CO$_2$ in air and cells were allowed to settle for 24 hrs. Anti-PSA antibodies at the dose of 0.5, 1, 2, 4, 8 and 16 $\mu$g per well in 40 $\mu$l medium were added. Control wells received 40 $\mu$l medium or preimmune serum reconstituted in medium.

In another set of culture, in addition to anti-PSA antibodies, 50 $\mu$l of diluted rabbit serum (1:10 in medium) previously demonstrated to have no cytotoxicity, was added to each well as source of complement. An unrelated antibody generated in goats against nuclear polyhedrosis baculovirus was added (16 $\mu$g/well in 40 $\mu$l medium) to a separate set of wells and served as antibody controls. Cultures were exposed to anti-PSA antibodies for 24, 48 and 72 hrs. at 37°C in a humidified atmosphere of 5% CO$_2$ in air. Cultures were pulsed with 1 $\mu$Ci $^3$H thymidine in 50 $\mu$l medium per well for 20-22 hrs. at 37°C. The cells were then harvested onto glass fibre filter strips using an automated cell harvester. The samples were counted for thymidine incorporation in a LKB $\beta$ counter. The % inhibition of thymidine incorporation was calculated by the following formula.

$$\% \text{ Inhibition} = 100 - \left( \frac{\text{Mean CPM of test wells}}{\text{Mean CPM of control wells}} \right) \times 100$$

3.8.1.2. b) Non-radioactive cell proliferation assay:

This assay origially described by Mosmann (1983) is based on the principle that the living cells convert the tetrazolium salt into a formazan product which upon solubilization has colour and is thus quantifiable. LNCaP, DU 145 and HEp-2 cells were grown separately in tissue culture flasks and
Materials and Methods

harvested using trypsin-EDTA solution at the log phase of growth. Cell suspensions (5 X 10^4 cells/ml) were made in media supplemented with FCS, penicillin and streptomycin mixture. 100 µl of this cell suspension was dispensed per well in 96 - well tissue culture plates. 40 µl of medium containing 0.5, 1, 2, 4, 8 or 16 µg of anti-PSA antibodies was added before incubation at 37°C in an atmosphere of 5% CO₂ in air for 24 hrs. 15 µl of dye solution (Cell Titre non-radio active cell proliferation assay kit - Promega) was then added per well and further incubation carried out at 37°C in a CO₂ incubator for 4 hrs. This was followed by overnight (10-12 hrs.) incubation after addition of 100 µl solubilization solution per well supplied in the kit. Absorbance was recorded at 570 nm by ELISA reader after a low speed shaking for 30 seconds.

3.8.2. In vivo studies:

3.8.2.1. Experimental animals:

Cytotoxic effect of anti-PSA antibodies was studied in transplantable DU 145 cells in nude mice where the cells grow into a tumour mass. Nude mice [NIH (SWISS) nu/nu] were procured from Laboratory Animals Information Services Centre (LAISC), National Institute of Nutrition (NIN), Hyderabad. Male mice of 6-8 weeks of age were used for the experiments. The animals were housed in sterile polypropylene cages with grilled stainless steel lids. Sterile paper shreds were provided as the bedding material. Standard balanced pelleted diet for mice from M/S Lipton India Ltd. was supplied sterile ad libitum as also filtered and autoclaved water supplemented with streptomycin (1.5 mg/ml) and bacitracin (1.5 mg/ml) or bacitracin alone (4 mg/ml). Bedding material, cages, feed and water bottles were changed every alternate day. Animal cages were housed in a laminar flow hood which
was positioned in an air cooled room with 12 hr. light and 12 hr. dark cycles. All changes, operational procedures, injections etc. were carried out aseptically inside the hood. Animals were examined once a day routinely for health and growth of transplantable tumour.

3.8.2.2. Implantation of cells for tumour growth:

DU 145 cells were used for in vivo experiments. In order to have a large number of cells these were grown in 75 culture flasks and the culture was expanded in T 150 culture flasks. Cells were harvested by trypsin (0.05%) - EDTA (0.02%) solution, and suspended in DMEM supplemented with 10% FCS and antibiotics (penicillin 100 IU/ml and streptomycin 100 μg/ml). HEp-2 cells grown similarly served as control tumours. Cells were injected subcutaneously in the mid-dorsal skin of mice using disposable needles and tuberculin syringes. Each mouse received 0.5 ml DMEM containing $1 \times 10^7$ cells. The growth of tumour from the implanted cells was monitored daily and the size recorded twice weekly using Vernier-calipers. Any notable observation pertaining to the growth of the tumour was recorded. The growth of tumours was also recorded by photography at regular intervals.

3.8.3. Inhibition of tumour growth:

DU 145 cells were incubated at 37°C in a CO₂ incubator for 1 hr. with 1, 5, 25 and 125 μg of anti-PSA immunoglobulins prior to subcutaneous implantations of three mice in each group. Simultaneously, anti-PSA antibodies at the indicated doses were injected intradermally close to the site of tumour growth twice per week. This treatment was continued for 14 weeks. Control tumour cells, HEp-2 were also exposed to 125μg anti-PSA
antibodies and incubated for one hour at 37°C in an incubator prior to implantation in mice and the same dose of antibody was passively administered twice weekly for 14 weeks.

3.8.4. Tumouricidal effect on growing tumours:

Effect of anti-PSA antibodies on the well grown DU 145 tumours was evaluated to ascertain the tumouricidal effect of the antibodies. In this set of experiments the tumours were grown in mice and the antibody treatment at different doses (0, 10, 100 and 500 μg/mouse) was initiated at 8 week time point. Three mice in each group received the indicated dose of anti-PSA antibodies intradermally close to the site of tumour growth twice weekly. The treatment was continued for 8 weeks. Three mice in the control group received normal saline.

3.8.5. Histology of tumour:

Animals from the 500 μg anti-PSA antibodies treated group and saline treated (control group) were sacrificed after 8 weeks of treatment. Tissues from the necrosed and control tumours were collected in 10% buffered formalin, dehydrated in ascending grades of propanol, embedded in paraffin wax and sectioned at 5 μm intervals. Sections were stained by hematoxylin and eosin stain and studied for histological features.

3.9. Studies with anti-LHRH antibody on the growth of prostatic carcinoma cells.

LNCaP and DU 145 cells were grown in tissue culture flasks harvested and cell suspensions (5X10⁴ cells/ml) made in media. 100 μl of cell suspension (5X10³ cells) was dispensed per well in 96-well tissue culture
plates. The culture plates were incubated at 37°C for 24 hours in a CO₂ incubator. The anti-LHRH IgG diluted in medium and 0.5, 2.5, 12.5 and 25 μg of antibody was dispensed into each well in 100 μl medium. Culture plates were incubated for 48 hrs. at 37°C in a humidified atmosphere of 5% CO₂ in air. The dye solution (15 μl/well) of non-radioactive cell proliferation assay kit (Promega) was added to each well and incubated for 4 hours at 37°C in a CO₂ incubator. This was followed by addition of solubilization solution (100μl/well) and overnight (10-12 hrs.) incubation at room temperature. Absorbance was recorded in an ELISA reader at 570 nm with slow shaking for 30 secs. For control, an unrelated IgG monoclonal antibody against baculovirus at the dose of 25 μg in 100 μl medium was used in a separate set of wells.